FORM PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEYS DOCKET NO. 4804SAB-1

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 35 CFR 1.5) 10/088627

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/ZA00/00173	18 September 2000	17 September 1999

TITLE OF INVENTION
"NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS"

APPLICANT(S) FOR DO/EO/US
BURTON, Stephanie Gail; DORRINGTON, Rosemary Ann and HARTLEY, Carol Janet

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. below concern documents or information included:

11. [ ] An Information Disclosure Statement under 37n CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.23 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

"EXPRESS MAIL" MAILING LABEL NUMBER: EV068097180US
DATE OF DEPOSIT: March 18, 2002

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, BOX PCT, WASHINGTON, D.C. 20231.

TYPED OR PRINTED NAME: JANICE MESSER

SIGNATURE: Janice Messer

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
10/088627	PCT/ZA00/00173	4804SAB-1

17. [X] The following fees are submitted:		CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):			
Search Report has been prepared by the EPO or JPO	$860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid USPTO	$1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$100.00		
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Surcharge of $130.00 for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	12 - 20 =	0	x $18.00	$ 0.00	
Independent Claims	3 - 3 =	0	x $80.00	$ 0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ $270.00	$	
	TOTAL OF ABOVE CALCULATIONS =			$ 1,000.00	
[] Applicant claims small entity status under 37 CFR 1.27.				$	
			SUBTOTAL =	$ 1,000.00	
Processing fee of $130.00 for furnishing the English translation later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
			TOTAL NATIONAL FEE =	$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **$40.00** per property +				$	
			TOTAL FEES ENCLOSED =	$ 1,000.00	
			Amount to be: refunded	$	
			charged	$	

a. [X] A check in the amount of $ 1,000.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-1970. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 cfr 1.494 or 1.495 has not been met, a petition to revive (37 cfr 1.137(a) or (b)) must be filed and granted to restore the application pending status.

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PATENT APPLICATIONS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

BURTON, et al.

Int'l. Serial No.: PCT/ZA00/00173

Int'l. Filing Date: 18 September 2000

Priority Date: 17 September 1999

For: "NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS"

Atty. File No.: 4804SAB-1

PRELIMINARY AMENDMENT

"EXPRESS MAIL" MAILING LABEL NUMBER: EV068097170US
DATE OF DEPOSIT: 3-18-02

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TYPED OR PRINTED NAME: JANICE MESSER

SIGNATURE: Janice Messer

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the initial review of the above-identified patent application by the Examiner, please enter the following Preliminary Amendments. Fees for this Preliminary Amendment are calculated and included with the Transmittal Letter accompanying this Amendment. Please charge any underpayment or debit any overpayment to Dseposit Account 19-1970.

Please amend the above-identified patent application as follows:

IN THE SPECIFICATION:

Please amend the specification at page 1 following the title to include the following paragraph:

This application claims the benefits under 35 U.S.C. § 365 of PCT International Application No. PCT/ZA00/00173 filed 18 September 2001 entitled "Microorganisms, Their Use and Methods for Producing D-Amino Acids" which was published in English on 22 March 2001 having International Publication Number WO 01/19982, and which claims priority to South African Patent No. ZA 99/5981 filed 17 September 1999.

IN THE CLAIMS:

Please cancel Claims 1-11 and add Claims 12-23 as follows:

12. A strain of an *Agrobacterium* sp. which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids.

13. The *Agrobactgerium* sp. as claimed in Claim 12, wherein said *Agrobactgerium* sp. constitutively expresses enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids in the absence of glutamine.

14. The *Agrobactgerium* sp. as claimed in Claim 12, wherein said *Agrobactgerium* sp. constitutively expresses enzymes which convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.

15. The *Agrobactgerium* sp. as claimed in claim 12 which is indistinguishable from *Agrobacterium* RU-OR based its 16S rRNA gene sequence.

16. A method of producing chemicals selected from the group consisting of pharmaceuticals, agrochemicals, pesticides and feedstock additives comprising producing said chemicals with an *Agrobactgerium* sp. as claimed in Claim 12.

17. An isolated enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids wherein said enzyme system is isolated from the *Agrobacterium* sp. as claimed in Claim 12.

18. An isolated enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids wherein said enzyme system is isolated from the *Agrobacterium* sp. as claimed in Claim 12.

19. A growth medium for use in the production of the *Agrobacterium* sp. as claimed in Claim 12.

20. The growth medium of Claim 19, wherein said growth medium causes over expression of an enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids in *Agrobacterium* sp. under fermentation conditions.

21. The growth medium of Claim 19, wherein said growth medium causes over expression of an enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids in *Agrobacterium* sp. under fermentation conditions.

22. A *N*-carbamylamino acid produced by a strain of an *Agrobacterium* sp. which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids.

23. A D-amino acid produced by a strain of an *Agrobacterium* sp. which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids.

REMARKS/ARGUMENTS

The above amendments are being submitted in connection with the national stage filing of the present Application. The amendments eliminate the multiple dependent claims from the Application.

Respectfully submitted,

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Date: 18 March 2002

NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS

FIELD OF THE INVENTION

The invention relates to novel micro-organisms and their use in the production of D-amino acids. In particular, micro-organisms suitable for the production of D-amino acids from corresponding hydantoins or *N*-carbamoylamino acids. These novel micro-organisms are simple to cultivate and make possible high D-amino acids yields from different substrates.

BACKGROUND OF THE INVENTION

The importance of optically pure amino acids is primarily due to the use of D-amino acids, e.g. D-*p*-hydroxyphenylglycine, as side chains in semi-synthetic penicillins and cephalosporins (Syldatk *et al*, 1990). Optically pure amino acids also have applications in the production of other pharmaceuticals and flavourants (e.g. D-alanine in sweetners), pesticides (D-valine in the synthesis of insecticide fluvanilate), and as additives in animal feedstock (Polastro, 1989). Conventionally, D, L-5-substituted hydantoins have been used as starting materials for the chemical synthesis of D-amino acids. This process is cumbersome and inefficient since chemical synthesis results in an equimolar mixture of D- and L-amino acids requiring racemate resolution to obtain optically pure D-amino acids (Syldatk *et al.*, 1990). An alternative to chemical synthesis is the use of enzymatic conversion of hydantoins to their respective amino acids (Olivieri *et al.*, 1979). Biocatalytic conversions have major advantages: the enzyme systems are stereoselective and mild reaction conditions

result in a cheap industrial process with environmentally benign by-products and effluents (Santaniello *et al*, 1992) The biocatalytic conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine has been listed as one of the main biocatalytic processes in the world market (Polastro, 1989).

The biocatalytic conversion of hydantoins to their corresponding amino acids is catalysed by two enzymes: first, an hydantoinase catalyses the ring-opening hydrolysis of the 5-substituted hydantoin to produce an *N*-carbamylamino acid in a reversible reaction. Classified as cyclic amidases (E.C.3.5.2), hydantoinases may be D-, L- or non-stereoselective. In the second reaction, the *N*-carbamylamino acid is converted to its corresponding amino acid either chemically, or through the action of a second enzyme, an *N*-carbamylamino acid amidohydrolase (E.C.3 5.1.6), which is usually stereoselective. (Olivieri *et al.*, 1979) While racemization of the hydantoins occurs spontaneously at alkaline pH, certain microbial systems include a D-racemase which converts L-5-substituted hydantoins to the corresponding D-enantiomers (Runser *et al.*, 1990; Hartley *et al.*, 1998).

D-selective hydantoin-hydrolysing enzyme systems have been identified in a variety of bacteria, including a *Pseudomonas* isolate (Ikenaka *et al.*, 1998), *Bacillus stearothermophilus* (Lee *et al.*, 1996), *Bacillus circulans* (Lukša *et al*, 1997) and several *Agrobacterium* strains (Olivieri *et al.*, 1981; Runser *et al.*, 1990; Hartley *et al.*, 1998; Nanba *et al.*, 1998). The genes encoding one hydantoinase and three *N*-carbamylamino acid amidohydrolase enzymes from the Agrobacterium strains have been cloned and over-expressed in *Escherichia coli* (Durham and Weber, 1995; Buson *et al.* 1996; Grifantini *et al.*, 1998; Nanba *et al.*, 1998). DNA sequence analysis has revealed a high degree of amino acid homology between *N*-carbamylamino acid amidohydrolases from the Agrobacteria (Nanba *et al.*, 1998).

Characterisation of the enzyme system of A. *tumefaciens* RU-OR showed that enzymes activity was induced at high levels only when cells were grown in the presence of 2-thiouracil or hydantoin. Furthermore, maximum enzyme activity in cells grown in complete medium was detected in early stationary phase. (Hartley *et al.*, 1988). Similar observations have been made for hydantoin-hydrolysing enzyme systems from *A. radiobacter* (Deepa *et al.*, 1993), *Agrobacterium* sp. IP I-671 (Meyer

& Runser, 1993) and those of other bacteria with L-selective enzyme systems, such as *Arthrobacter crystallopoietes* (Möller *et al.*, 1988) An *A. tumefaciens* mutant, with inducer-independent production of hydantoinase and NCAAH, has been isolated by Hartley *et al.* (1998) and a similar mutant strain, *Arthrobacter* sp. DSM 9771, has been isolated by Wagner *et al.* (1996).

In this invention the word "constitutive" is to be understood to mean unregulated expression of enzymes; the word "expression" is understood to mean the production of a protein from a DNA template via transcription and translation; the word "activity" is understood to mean the ability of the hydantoinase and *N*-carbamylamino acid aminohydrolase enzymes to hydrolyse hydantoins to *N*-carbamylamino acids and amino acids and vice versa, respectively, the phrase "over-express" to mean levels of enzyme production in excess of those under the same conditions in the original isolate, and the phrase "enzyme system" is to be understood to include hydantoinase, *N*-carbamylamino acid amidohydrolase and hydantoin racemase enzymes which are capable of converting D- or L- or D,L-5-monosubstituted hydantoins or D- or L- or D,L- *N*-carbamoylamino acids to their corresponding, optically pure D-amino acids.

Recombinant systems for the over-expression of both hydantoinase and NCAAH enzymes in *E. coli* are known. However, reports of the production of insoluble aggregates and plasmid instability in cells over-expressing the NCAAH indicate that heterologous expression of these enzymes in *E. coli* may not be the ideal system. This has led to renewed interest in the use of homologous hosts for hydantoinase and NCAAH production, where the main problem is that enzyme activity needs to be induced and is confined to stationary growth phase under optimum growth conditions. This means that the levels of enzyme production per unit biomass in commercial strains remain relatively low. The re-introduction of a recombinant NCAAH gene under control of a constitutive promoter into *Agrobacterium* 80/44-2A resulted in high levels of biocatalytic activity.

The problems relating to genetically modified organisms and the obvious economic advantages of industrial strains that are not genetically modified, have led to the examination of the potential of mutant bacterial strains in the high-level production of hydantoinase and NCAAH enzymes.

OBJECT OF THE INVENTION

An object of the invention is the isolation of micro-organisms able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins or N-carbamyl amino acids to D-amino acids and thereby, at least partially, to alleviate the problems associated with chemical synthesis of D-amino acids

SUMMARY OF THE INVENTION

In accordance with the invention there is provided a biologically pure culture of a mutant strain of *Agrobacterium* RU-OR which constitutively expresses a stereoselective enzyme system which may be used in the enzymatic synthesis of D-amino acids.

Further in accordance with the invention there is provided a biologically pure culture of a glutamine synthesis-deficient micro-organism able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids.

Furthermore in accordance with the invention there are provided micro-organisms which are able to constitutively produce enzymes which convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.

Further in accordance with the invention there is provided an isolated and purified enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids.

Still further in accordance with the invention there is provided an isolated and purified enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.

Furthermore in accordance with the invention there is provided a micro-organism for use in the production of D-amino acids for the production of pharmaceuticals,

alternatively agrochemicals, further alternatively for use in the production of D-amino acids for the production of pesticides. and still further alternatively for use in the production of D-amino acids for the production of feedstock additives.

The invention also extends to a growth medium to achieve over-expressed levels of hydantoinase and/or NCAAH enzyme activity during optimum culture conditions.

The invention also provides for a *N*-carbamylamino acid produced in accordance with the invention.

The invention also provides for a D-amino acid produced in accordance with the invention

BRIEF DESCRIPTION OF THE FIGURES

In the accompanying Figures:

Figure 1 shows the DNA sequence of the 16S rRNA gene of *Agrobacterium* RU-OR;

Figure 2 shows hydantoinase and *N*-carbamylamino acid amidohydrolase activity in *Agrobacterium* RU-OR cells during mid-logarithmic phase during growth in HMM,

Figure 3 shows the effect of carbon and nitrogen source on hydantoinase and *N*-carbamylamino acid amidohydrolase activities in RU-OR cells;

Figure 4 shows that ammonia shock represses enzyme activity in wild-type *Agrobacterium* RU-OR cells;

Figure 5 shows that RU-ORPN1 cells constitutively express hydantoinase enzyme, but that the hydantoinase enzyme is inactive due to repression by ammonium in the growth medium;

Figure 6 shows that RU-ORPN1 cells constitutively express active *N*-carbamylamino acid amidohydrolase enzyme, while the wild type enzyme is repressed;

Figure 7 shows that hydantoinase activity in RU-ORPN1F9 cells is not sensitive to ammonia shock;

Figure 8 shows the levels of hydantoinase activity in RU-ORPN1F9 cells during mid-logarithmic growth phase compared with the levels in the wild-type RU-OR and mutant RU-ORPN1, when cells are grown under optimal growth conditions;

Figure 9 shows the levels of *N*-carbamylamino acid amidohydrolase activity in both RU-ORPN1 and RU-ORPN1F9 cells during mid-logarithmic growth phase compared with the levels in the wild-type RU-OR, when cells are grown under optimal growth conditions, and

Figure 10 shows the increase in specific hydantoinase activity per unit biomass in RU-ORPN1F9 cells in mid-logarithmic growth phase, with D,L-*p*-hydroxyphenylhydantoin as substrate, as compared with the specific hydantoinase activity in the wild-type RU-OR cells and RU-ORPN1 cells achieved during stationary phase.

DESCRIPTION OF ONE EMBODIMENT OF THE INVENTION

Several *Agrobacterium* strains have been reported to have hydantoin-hydrolysing activity. Among these are *Agrobacterium tumefaciens* 47 C, *Agrobacterium radiobacter* B11291 and *Agiobacterium* sp. IP I-671 *Agrobacterium radiobacter* B11291 and *Agrobacterium* sp IP I-671 also have *N*-carbamylamino acid and amidohydrolase activity. In the present invention, a novel *Agrobacterium* species (RU-OR) was isolated which is capable of producing a number of enzymes in amounts such that the cell mass has a high activity for the methods described herein.

CULTURE AND BIOCATALYTIC ASSAY CONDITIONS

Agrobacterium RU-OR and RU-ORPN1 cells grown to saturation in hydantoin minimal medium (HMM) broth. are diluted to OD_{600nm} = 0.02 in standard minimal

medium (MM) (MM per litre: 10g glucose; 0.011g $CaCl_2$, 0.02g $MgCl_2$; 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 0.04g boric acid, 0.04g $MnSO_4$, 0.02g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.01g KI, 0.004g $CuSO_4$) supplemented with 1% hydantoin (HMM), 0.01% casamino acids (SMM), or $(NH_4)_2SO_4$ (AMM). Strain RU-ORPN1F9 cells are grown in HMM or SMM or AMM supplemented with 0.002% glutamine. Enzyme activity in *Agrobacterium* RU-OR cells was induced by growth in medium containing 0.1% thiouracil. Cells are harvested at OD_{600nm} = 0.5 – 0.8, pelleted by centrifugation, washed in 0.1 M PO_4 buffer pH 8.0 and resuspended in hydantoin or *N*-carbamylglycine reaction buffer at a final hydrated biomass concentration of 20 mg/ml (reaction buffer: either 50 mM hydantoin or 25 mM *N*-carbamylglycine in 0.1 M PO_4 buffer pH 8.0). Hydantoinase activity is measured as the sum of the concentration of *N*-carbamylglycine (µmol/ml) and glycine (µmol/ml) produced from 50 µmol/ml hydantoin in a 5 ml reaction volume after 6 h, shaking, at 40°C. *N*-carbamylamino acid amidohydrolase activity is measured as the concentration of glycine (µmol/ml) produced from 25 µmol/ml *N*-carbamylglycine in a 5 ml reaction volume after 6 h, shaking, at 40°C.

ISOLATION OF *AGROBACTERIUM* RU-OR, RU-ORPN1 and RU-ORPN1F9

Soil samples from the Eastern Cape environment were inoculated into hydantoin minimal medium (HMM) broth (per litre: 10g glucose; 0.011g $CaCl_2$; 0.02g $MgCl_2$; 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 0.04g boric acid, 0.04g $MnSO_4$ 0.02g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.01g KI, 0.004g $CuSO_4$, 1% hydantoin) and incubated, shaking at 25°C for 24 hours, after which serial dilutions were plated onto HMM agar and incubated for 5 days at 25°C. Resulting colonies, which utilised hydantoins as a sole nitrogen source, were purified by re-streaking onto HMM agar. Isolated strains were examined for the presence of hydantoinase and *N*-carbamylamino acid amidohydrolase activity using resting cell biocatalytic assays. The wild-type *Agrobacterium* sp strain RU-OR, which was among these isolates, was identified through determination of its 16S rRNA gene sequence (shown in Figure 1) as described in Hartley *et al.* (1998).

Mutant RU-ORPN1 was selected as follows: *Agrobacterium* RU-OR cells were cultured in HMM broth to mid-log phase and then subjected to mutagenesis using

ethylmethane sulfonate (EMS) according to the method described in Miller (1992). Mutated cells were plated onto MM agar supplemented with 0.1% $(NH_4)_2SO_4$ and 0.1% 5-fluorouracil. Strain RU-ORPN1 was isolated from these plates and evaluated under standard culture and assay conditions for enzyme activity in the absence of inducer. Strain RU-ORPN1F9 was isolated by mutagenizing RU-ORPN1 cells as described above and after penicillin-enrichment for glutamine-dependent growth, cells were plated onto HMM agar supplemented with 0 002% glutamine. *Gln*$^-$ mutants were selected by relica plating to HMM without supplementation with glutamine.

GLUTAMINE SYNTHETASE ASSAYS.

Total glutamine synthetase activity was measured using the γ-glutamyl transferase assay. Cells were prepared by treatment with 0.01% cetyl-trimethylammonium bromide for 10 minutes before harvesting. The cells were then washed twice with 0.1M phosphate buffer pH 9.0 before being suspended in 50 times less volume of resuspension buffer, and assayed according to the method of Bender *et al.* (1977). Protein concentration was determined by the method of Bradford (1976). Activity is expressed as μmoles of γ-glutamyl hydroxamate generated per minute per milligram protein. The percentage adenylation of the glutamine synthetase enzyme subunits was measured using the method of Magasanik *et al.* (1995), which compares γ-glutamyl transferase in the presence and absence of magnesium ions Magnesium ions inhibit the activity of adenylated enzyme subunits and the difference can then be used to calculate the percentage adenylation of the glutamine synthetase enzyme.

REGULATION OF HYDANTOINASE AND NCAAH ACTIVITY

Hydantoinase and NCAAH activities in *A. tumefaciens* RU-OR cells could be detected only in early stationary phase during batch culture in a complete growth medium (nutrient broth). Furthermore, enzyme activity was dependent upon growth in the presence of the hydantoin-analogue 2-thiouracil. The nutritional factors responsible for regulating enzyme activity were identified by establishing standard culture conditions under which enzyme activity was not limited to stationary phase Hydantoinase and NCAAH activities were measured during growth of RU-OR cells in a chemically defined minimal medium containing hydantoin and glucose as sole

nitrogen and carbon sources, respectively (MM plus 0 1 % hydantoin). Activity of both enzymes was low in early exponential phase and after the cells reached stationary phase, with highest activity detected during mid to late exponential phase (Figure 2).

In all subsequent experiments, enzyme activities were determined in cells harvested during mid-exponential phase at OD_{600} = 0 5 - 0.8

The effect of different carbon and nitrogen sources upon hydantoin-hydrolysing enzyme activity was determined by examining growth-rate and assaying for biocatalytic activity at mid-exponential growth phase. Cells were grown in minimal medium containing either glucose or glycerol as carbon source and hydantoin as nitrogen source. The growth-rate of RU-OR cells was not significantly affected by either carbon source (Figure 3) and there was also little difference in hydantoinase and NCAAH activity (Table 1)

Table 1. Hydantoin-hydrolysing activity in RU-OR cells grown with different carbon and nitrogen sources.

Carbon Source	Nitrogen Source	Hydantoinase Activity (µmol/ml)	NCAAH Activity (µmol/ml)
1% glucose	1 % hydantoin	4.87 ± 0.400	5.77 ± 0.55
1% glycerol	1 % hydantoin	3.97 ± 0.58	5.85 ± 0.58
1% glucose	0.1% $(NH_4)_2SO_4$	1.15 ± 0.2	1.09 ± 0.16
1% glucose	0.1% serine	4.70 ± 0.26	3.70 ± 0.56*
1% glucose	0.01% CAA	10 87 ± 0.43	8.68 ± 0.61

± - SEM (n = 3). * Measured as the amount of glycine generated from hydantoin as substrate. CAA - casamino acids.

In contrast, the growth rate of RU-OR cells appeared to be dramatically affected by the choice of nitrogen source. Hydantoin was the most growth-rate-limiting while 0.1% $(NH_4)_2SO_4$ and 0.1% serine were the least growth-rate limiting sources of nitrogen (Figure 3). Cells in medium containing 0.01% casamino acids, grew at an intermediate rate. The highest enzyme activity was detected in cells growing in

0 01% casamino acids and the lowest in $(NH_4)_2SO_4$. Cells grown with serine or hydantoin as a nitrogen source showed intermediate levels of enzyme activity (Table 1): growth of cells in medium containing $(NH_4)_2SO_4$ had a repressive effect upon hydantoinase and NCAAH activity (nitrogen repression).

Induced RU-OR cells (grown in SMM plus 0.1% thiouracil) were resuspended and grown in AMM plus 2-thiouracil (ammonia shock). Within 30 minutes, the hydantoinase activity had dropped three-fold, and a corresponding two-fold drop in NCAAH activity was observed (Figure 4).

When induced cells were resuspended and grown in AMM containing the glutamine synthetase inhibitor, D,L-methionine D,L-sulfoximine (MSX), there was very little drop in both hydantoinase and NCAAH activities (Figure 4), indicating that the loss of hydantoinase and NCAAH activity in RU-OR cells after ammonia shock is dependent upon glutamine synthetase activity. Induced cells were subjected to ammonia shock for 30 minutes, after which they were washed and resuspended in SMM plus thiouracil and grown for a further 60 minutes before assaying for enzyme activity. Hydantoinase and NCAAH activity returned to levels observed before ammonia shock suggesting that the ammonia shock effect could be reversed rapidly in the absence of $(NH_4)_2SO_4$. Together, this data indicates that hydantoinase and NCAAH activity in wild-type *Agrobacterium* RU-OR is dependent upon the presence of a) inducer and b) the nitrogen source in the growth medium.

CHARACTERIZATION OF MUTANT STRAINS.

Inducer-independent hydantoinase and *N*-carbamylamino acid amidohydrolase, activity was assessed by measuring enzyme activity in cells grown in SMM without 2-thiouracil. RU-ORPN1 cells showed a significant (three-fold) increase in hydantoinase activity and NCAAH activity was equivalent to induced levels in *Agrobacterium* RU-OR cells

Table 2. Hydantoin-hydrolysing activity of mutant RU-OR strains

Strain	HYDANTOINASE *N*-carbamylglycine plus glycine (µmol/ml)		NCAAH Glycine (µmol/ml)	
	no inducer	2-thiouracil	no inducer	2-thiouracil
RU-OR (wt)	1.98±0 65	7 51±0 37	2.62±0.15	11.74±0.80
RU-ORPN1	21.8±0.78	nd	8.04±0.35	nd

± - SEM (n = 3). nd – not determined.

RU-ORPN1 cells grown in minimal medium with $(NH_4)_2SO_4$ as nitrogen source had repressed levels of hydantoinase activity, as observed in the wild-type, RU-OR cells (Figure 5), but, in contrast to the RU-OR, NCAAH activity in RU-ORPN1 cells was elevated to wild-type, induced levels (Figure 6). After growth in SMM for 60 minutes, hydantoinase activity in mutant RU-ORPN1 cells recovered to levels normally observed in induced wild-type cells (see table 2) while there was no increase in hydantoinase activity in the wild-type *Agrobacterium* RU-OR cells after growth in SMM Thus, unlike the wild-type, the mutant strain expresses both hydantoinase and *N*-carbamylamino acid amidohydrolase enzymes even under nitrogen repression conditions, but the hydantoinase enzyme is inactive in the presence of $(NH_4)_2SO_4$.

Inhibition of glutamine synthesis reduces the sensitivity of hydantoinase activity to ammonia shock in RU-OR cells (Figure 4). Therefore, the *gln*⁻ auxotrophic mutant RU-ORPN1F9 was subjected to ammonia shock and hydantoinase activity in the auxotrophic mutant. Figure 7 shows that hydantoinase activity in mutant RU-ORPN1F9 is no longer sensitive to ammonia shock as compared to that of the wild-type *Agrobacterium* RU-OR and mutant RU-ORPN1

Glutamine synthetase assays of all three strains before and after ammonia shock showed that glutamine synthesis was reduced by 60% in RU-ORPN1F9 when compared to that in *Agrobacterium* RU-OR and RU-ORPN1 cells. Thus a reduction in glutamine synthesis when RU-ORPN1F9 cells are grown in $(NH_4)_2SO_4$, results in insensitivity of hydantoinase activity to ammonia shock.

HYDANTOINASE AND NCAAH ACTIVITY IN REGULATORY MUTANTS DURING GROWTH IN $(NH_4)_2SO_4$.

The hydantoinase and NCAAH activity of RU-ORPN1 and RU-ORPN1F9 cells were assessed during batch culture in SMM and compared with enzyme activity of the wild-type *Agrobacterium* RU-OR grown in the same medium, supplemented with 2-thiouracil.

Hydantoinase activity in mutant strain RU-ORPN1 followed the same trend as in the wild-type *Agrobacterium* RU-OR (Figure 8), but high levels of activity were detected in exponential growth phase in RU-ORPN1F9 cells NCAAH activities in strains RU-ORPN1 and RU-ORPN1F9 were highest in exponential growth phase and these levels declined during stationary phase. RU-ORPN1F9 cells achieved the highest overall hydantoin-hydrolyzing activity of all three strains during exponential growth phase (Figures 8 and 9) indicating that the *gln*$^-$ phenotype does not have a deleterious effect upon hydantoinase or NCAAH production in this strain. Strain *Agrobacterium* RU-OR was selected for its efficient conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine. High levels of D,L-*p*-hydroxyphenylhydantoin-hydrolysis were also achieved. The highest D,L-*p*-hydroxyphenylhydantoin conversion by the wild-type *Agrobacterium* RU-OR and RU-ORPN1 cells was detected during stationary growth phase. In strain RU-ORPN1F9 both hydantoinase and NCAAH activity during exponential growth phase exceeded that detected in either *Agrobacterium* RU-OR or RU-ORPN1 cells Up to 45 % of D,L-*p*-hydroxyphenylhydantoin was converted to either *N*-carbamyl-*p*-hydroxyphenylglycine or D-*p*-hydroxyphenylglycine by RU-ORPN1F9 cells within six hours. RU-ORPN1F9 cells produced approximately 6 µmoles/ml D-*p*-hydroxyphenylglycine after six hours, which corresponds to 25 % conversion of D,L-*p*-hydroxyphenylhydantoin.

Figure 10 (A – C) depicts the specific hydantoinase activity per milligram dry cell mass with D,L-*p*-hydroxyphenylhydantoin as substrate. Strain RU-ORPN1 shows an overall increase of 50% in hydantoinase activity compared with wild-type *Agrobacterium* RU-OR. Mutant RU-ORPN1F9 showed the highest specific

hydantoinase activity with a 300% and 200% increase over the wild-type *Agrobacterium* RU-OR and mutant RU-ORPN1 respectively Most important, the highest specific hydantoinase activity per unit biomass was observed in RU-ORPN1F9 cells during mid-logarithmic growth phase (0.015 units) versus 0.002 units and 0 003 units of activity in RU-OR and RU-ORPN1 cells, respectively, during the same growth phase.

CLAIMS

1. A biologically pure culture of a mutant strain of micro-organism which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids
2. A biologically pure culture glutamine deficient micro-organism able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids.
3. A micro-organism able constitutively to produce enzymes which convert racemic mixtures of N-carbamylamino acids to D-amino acids.
4. A micro-organism able constitutively to produce enzymes which convert racemic mixtures of N-carbamylamino acids to D-amino acids.
5. A micro-organism as claimed in any one of claims 1 to 3 wherein the micro-organism is *Agrobacterium* sp.
6. A micro-organism as claimed in any one of claims 1 to 4 wherein the micro-organism is indistinguishable from *Agrobacterium* RU-OR based on its 16S rRNA gene sequence.
7. An isolated and purified enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids where the enzyme system is isolated and purified from a micro-organism as claimed in any one of claims 1 to 3.
8. An isolated and purified enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids where the enzyme system is isolated and purified from a micro-organism as claimed in any one of claims 1 to 3.
9. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of pharmaceuticals.
10. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of agrochemicals.
11. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of pesticides
12 A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of feedstock additives.

13. A growth medium for use in the production of a micro-organism constitutively producing an enzyme system catalysing the conversion of 5-substituted hydantoins to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

14. A growth medium for use in the production of a micro-organism constitutively producing an enzyme system catalysing the conversion of *N*-carbamylamino acids to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

15. A growth medium for use in the production of micro-organisms as claimed in any one of claims 1 to 4 producing an enzyme system as claimed in either one of claims 5 or 6.

16. A growth medium as claimed in any one of claims 1 to 13 for the production of D-Amino acids from 5-substituted hydantoins during fermentation conditions.

17. A growth medium as claimed in any one of claims 1 to 13 for the production of D-Amino acids from *N*-carbamoylamino acids during fermentation conditions.

18. A growth medium for use under fermentation conditions to achieve over-expressed levels of enzyme activity for the conversion of racemic mixtures of 5-substituted hydantoins to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

19. A growth medium for use under fermentation conditions to achieve over-expressed levels of enzyme activity for the conversion of racemic mixtures of *N*-carbamylamino acids to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

20. A *N*-carbamylamino acid produced in accordance with the invention.

21. A D-amino acid produced in accordance with the invention.

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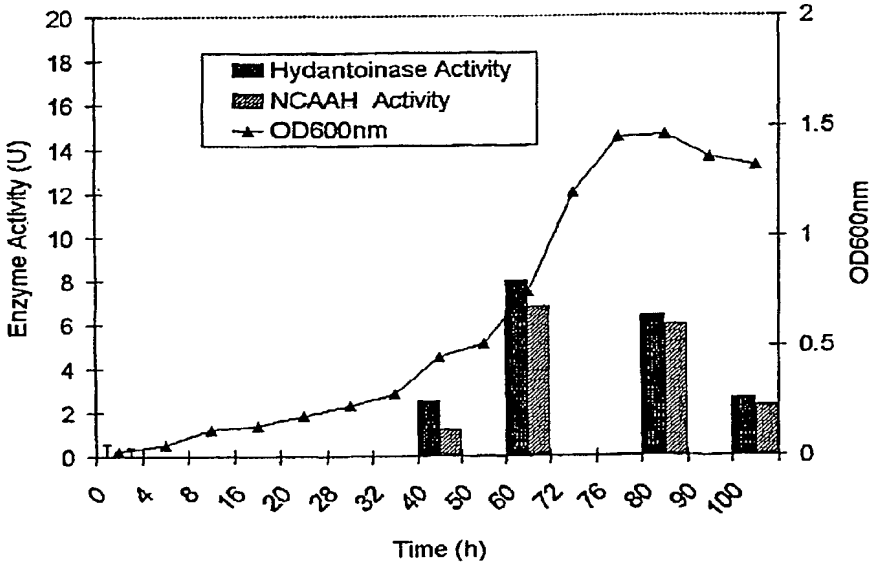
[Continued on next page]

(54) Title: NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS



(57) Abstract: The invention relates to novel micro-organisms which are simple to cultivate and their use in the production of D-amino acids, particularly micro-organisms suitable for the production of D-amino acids from corresponding hyantoins of *N*-carbamoylamino acids.

```
1    CCGCAAGGGA GTGGCAGACG GGTGAGTAAC GCGTGGGAAC ATACCCTTTC
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351  GACGGTATCC GGAGAAGAAG CCCCGGCTAA CTTCGTGCCA GCAGCCGCGG
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701  TAGCCGTCGG GCAGTATACT GTTCGGTGGC GCAGCTAACG CATTAAACAT
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751  TCCGCCTGGG GAGTACGGTC GCAAGATTAA AACTCAAAGG AATTGACGGG
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801  GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGCAGA
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851  ACCTTACCAG CTCTTGACAT TCGGGGTATG GGCATTGGAG ACGATGTCCT
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901  TCAGTTAGGC TGGCCCCAGA ACAGGTGCTG CATGGCTGTC GTCAGCTCGT
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1101    ACACACGTGC TACAATGGTG GTGACAGTGG GCAGCGAGAC AGCGATGTCG
        TGTGTGCACG ATGTTACCAC CACTGTCACC CGTCGCTCTG TCGCTACAGC

1151    AGCTAATCTC CAAAAGCCAT CTCAGTTCGG ATTGCACTCT GCAACTCGAG
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1201    TGCATG
        ACGTAC
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Figure 1

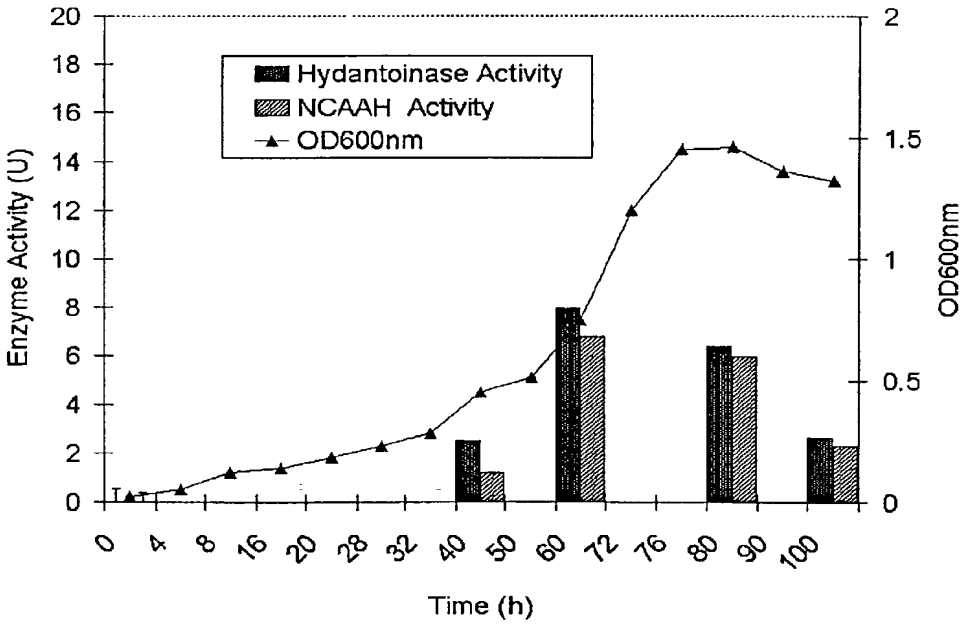
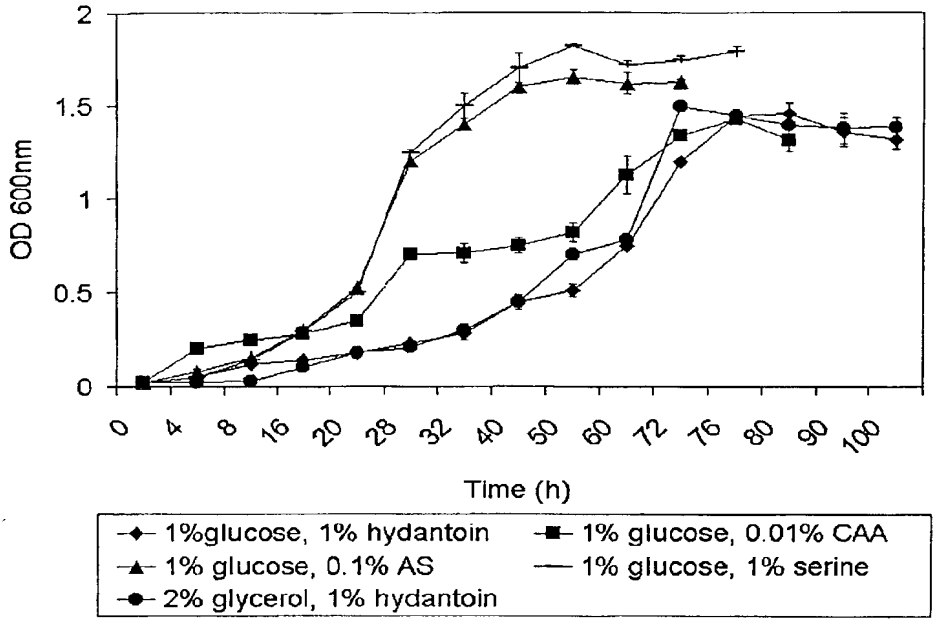


Figure 2



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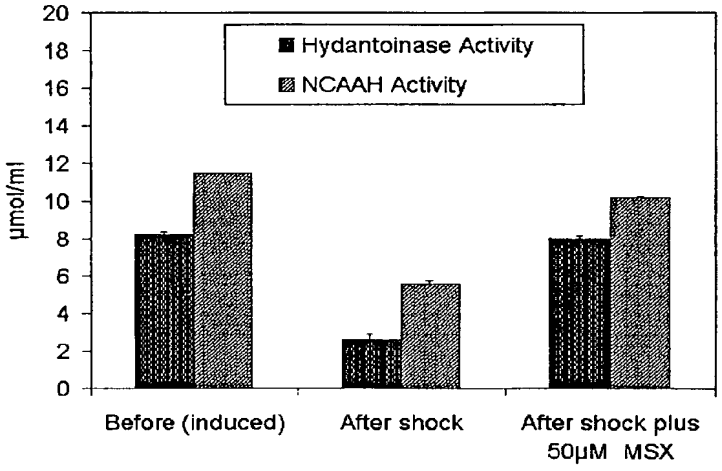

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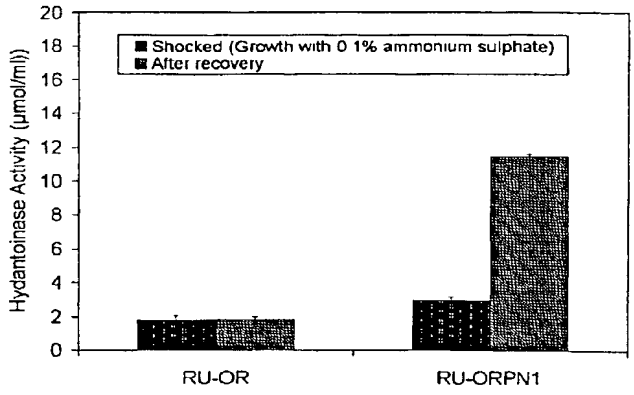
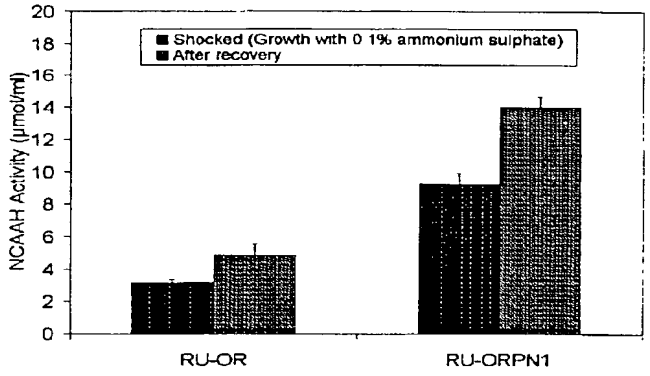


Figure 5



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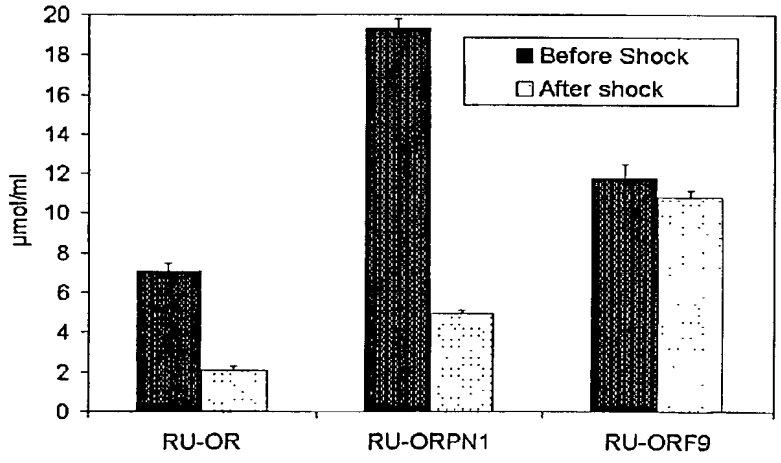

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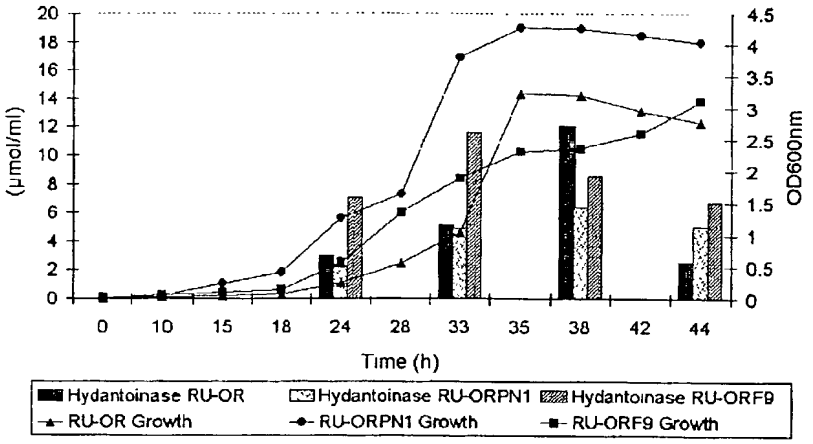

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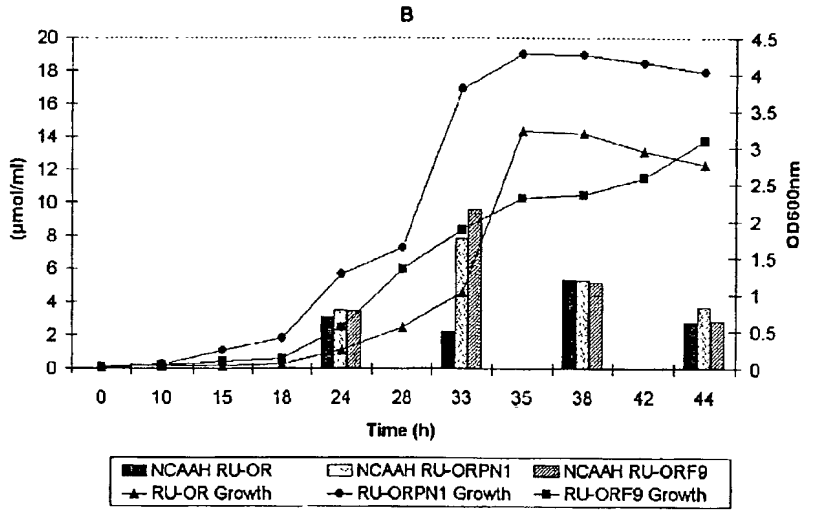

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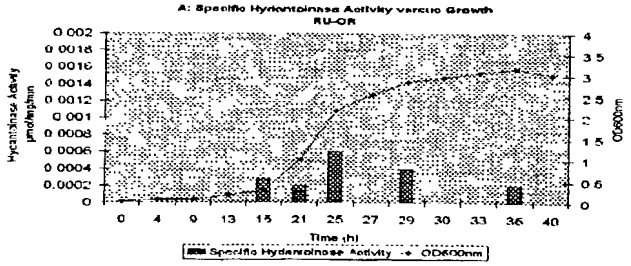
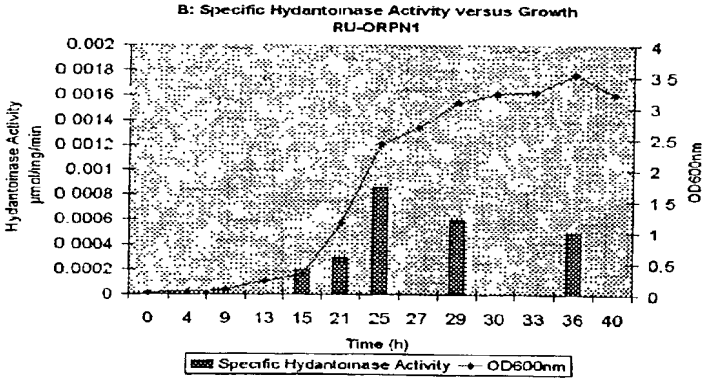

A: Specific Hydantoinase Activity versus Growth
RU-OR
Hydantoinase Activity
μmol/mg/min
OD600nm
0 002
0 0018
0 0016
0 0014
0 0012
0 001
0 0008
0 0006
0 0004
0.0002
0
4
3 5
3
2 5
2
1 5
1
0 6
0
0 4 9 13 15 21 25 27 29 30 33 36 40
Time (h)
Specific Hydantoinase Activity
OD600nm


B: Specific Hydantoinase Activity versus Growth
RU-ORPN1
Hydantoinase Activity
μmol/mg/min
OD600nm
0.002
0 0018
0 0016
0 0014
0 0012
0.001
0 0008
0.0006
0 0004
0.0002
0
4
3.5
3
2.5
2
1.5
1
0.5
0
0 4 9 13 15 21 25 27 29 30 33 36 40
Time (h)
Specific Hydantoinase Activity
OD600nm

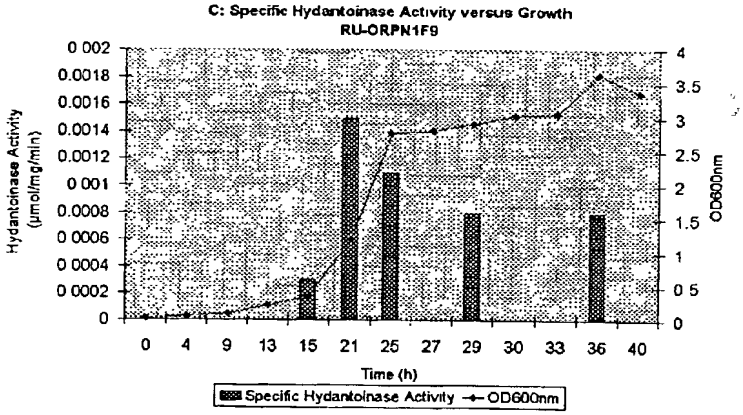

C: Specific Hydantoinase Activity versus Growth
RU-ORPN1F9
Hydantoinase Activity
(μmol/mg/min)
OD600nm
0 002
0.0018
0.0016
0.0014
0.0012
0 001
0.0008
0 0006
0 0004
0 0002
0
4
3.5
3
2.5
2
1.5
1
0 5
0
0 4 9 13 15 21 25 27 29 30 33 36 40
Time (h)
Specific Hydantoinase Activity
OD600nm

Figure 10

PTO/SB/01 (6-95)
Approved for use through 10/31/98 OMB 0651-0032

Type a plus sign (+) inside this box → ☐

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing

Attorney Docket Number	4804SAB-1
First Named Inventor	BURTON, Stephanie Gail
COMPLETE IF KNOWN	
Application Number	10/088,627
Filing Date	
Group Art Unit	
Examiner Name	

As below named inventor, I hereby declare that::

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for which a patent is sought on the invention entitled:

"NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS"

(*Title of the Invention*)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 18 September 2000 as United States Application Number or PCT International Application Number PCT/ZA00/00173 and was amended on (MM/DD/YYYY) _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1 56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any Pct international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? Yes	Certified Copy Attached? No
99/5981	South Africa	September 17, 1999	☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)		
		☐	Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States of PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112.1 acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (*if applicable*)

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As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☒ Firm Name OR SHERIDAN ROSS P.C. Customer Number or label 22442

☐ List attorney(s) and/or agent(s) name and registration number below:

Name	Registration Number	Name	Registration Number
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GROSETH, CRAIG C.	31,713	KOVARIK, JOSEPH E.	33,005
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CONNELL, GARY J	32,020	KUGLER, BRUCE A.	38,942
CROOK, WANNELL M.	31,071	BRUNELLI, ROBERT R.	39,617
STAVISH, SABRINA CROWLEY	33,374	JOHNSON, Brent P.	35,031

☒ Additional attorney(s) and/or agent(s) named on a supplemental sheet attached hereto

Please direct all correspondence to: ☐ Customer Number or label | OR ☐ Fill in correspondence address below

Name	Robert D. Traver				
Address	1560 BROADWAY, SUITE 1200				
Address					
City	DENVER	State	COLORADO	Zip	80202-5141
Country	UNITED STATES OF AMERICA	Telephone	(303) 863-9700	Fax	(303) 863-0223

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name	Stephanie	Middle Initial	G.	Family Name	Burton	Suffix e.g. Jr.	

Inventor's Signature	Stephanie G. Burton	Date	7.04.02

Residence City	Rondebosch	State	South Africa	Country	ZA	Citizenship	ZA

Post Office Address	c/o Department of Chemical Engineering University of Cape Town ZAF
Post Office Address	

City	Rondebosch	State	South Africa	Zip	7701	Country	ZA	Applicant Authority	

☒ Additional inventors are being named on supplemental sheet(s) attached hereto.

Type a plus sign (+) inside this box → ☐

DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet

Name of Additional Joint Inventor, if any:				☐ A petition has been filed for this unsigned inventor			
Given Name: Rosemary	Middle Initial: A.	Family Name: Dorrington	Suffix e.g. Jr.				
Inventor's Signature: RAD	Date: 5 April 2002						
Residence City: Grahamstown	State: South Africa	Country: ZA	Citizenship: ZA				
Post Office Address: c/o Department of Biochemistry and Microbiology Rhodes University, Biological Sciences Building Prince Alfred Street ZAF							
Post Office Address:							
City: Grahamstown	State: South Africa	Zip:	Country: ZA	Applicant Authority:			

Name of Additional Joint Inventor, if any:				☐ A petition has been filed for this unsigned inventor			
Given Name: Carol	Middle Initial: J.	Family Name: Hartley	Suffix e.g. Jr.				
Inventor's Signature: JMarkay	Date: 5 April 2002						
Residence City: Grahamstown	State: South Africa	Country: ZA	Citizenship: ZA				
Post Office Address: c/o Department of Biochemistry and Microbiology Rhodes University, Biological Sciences Building Prince Alfred Street ZAF							
Post Office Address:							
City: Grahamstown	State: South Africa	Zip:	Country: ZA	Applicant Authority:			

Name of Additional Joint Inventor, if any:				☐ A petition has been filed for this unsigned inventor			
Given Name:	Middle Initial:	Family Name:	Suffix e.g. Jr.				
Inventor's Signature:	Date:						
Residence City:	State:	Country:	Citizenship:				
Post Office Address:							
Post Office Address:							
City:	State:	Zip:	Country:	Applicant Authority:			

Name of Additional Joint Inventor, if any:				☐ A petition has been filed for this unsigned inventor			
Given Name:	Middle Initial:	Family Name:	Suffix e.g. Jr.				
Inventor's Signature:	Date:						
Residence City:	State:	Country:	Citizenship:				
Post Office Address:							
Post Office Address:							
City:	State:	Zip:	Country:	Applicant Authority:			

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet

See Notes to the request form

Type a plus sign (+) inside this box → ☐

DECLARATION	ADDITIONAL and/or AGENT INFORMATION Supplemental Sheet

Name	Registration Number	Name	Registration Number
CARDWELL, DANA HARTJE	40,638		
DALLAS, ANGELA K.	42,460		
LIEB, BENJAMIN B.	42,801		
KNEPPER, BRADLEY M.	44,189		
TRUDELL, MIRIAM DRICKMAN	42,499		
DuPRAY, DENNIS J.	46,299		
WINTERTON, Kenneth C.	48,040		
TRAVER, Robert D.	47,999		
YASKANIN, Mark L.	45,246		
KOCIALSKI, Mollybeth R.	42,754		